Characterization of 5′-regulatory region of human myostatin gene: regulation by dexamethasone in vitro

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Myostatin, previously known as growth differentiation factor-8 and a suggested member of the transforming growth factor superfamily, is predominantly expressed in skeletal muscle throughout life, from the early stages of embryogenesis to late adulthood (30). It was first identified by McPherron and Lee (30), who observed that gene targeting that disrupted myostatin expression resulted in a dramatic increase in the skeletal muscle mass of null mice. Subsequently, naturally occurring mutations that inactivate myostatin were identified in Belgian Blue and Piedmontese cattle (20), two breeds that are conspicuous because of their hypermuscularity (30). Conversely, increased myostatin expression was found to be associated with certain physiological conditions that result in a loss of muscle mass, such as acquired immune deficiency syndrome wasting syndrome (16), exposure to microgravity during space flight (22, 45), and hindlimb suspension (8, 46). Collectively, these data suggest that myostatin may act as an inhibitor of skeletal muscle growth.

The maintenance of muscle mass is determined by a fine balance between protein synthesis and breakdown, a dynamic homeostatic state modulated by numerous anabolic and catabolic factors. Muscle atrophy resulting from catabolic factors, such as glucocorticoids, starvation, and illness, could be the result of inhibition of protein synthesis or stimulation of protein breakdown in skeletal muscle (17, 18). Conversely, anabolic factors, such as androgens, recombinant human growth hormone, and resistance exercise, increase protein synthesis and thus promote muscle hypertrophy (2, 11). The precise processes by which these factors affect skeletal muscle growth or influence myostatin gene expression remain unclear.

As a first step toward elucidating the mechanisms that regulate myostatin gene expression, we cloned the 5′-upstream regulatory region of the human myostatin gene and characterized the regulatory elements present. Deletion analysis of the promoter was then used to further identify which of these elements was necessary for basal transcriptional activity of myostatin in vitro.

Furthermore, because the myostatin promoter region was found to contain many putative glucocorticoid response elements, we considered the possibility that glucocorticoids may regulate myostatin gene expression. Therefore, we first investigated the effects of dexamethasone (a glucocorticoid agonist) on myostatin transcriptional activity by using myostatin promoter luciferase reporter gene constructs. Subsequently, we examined the effects of graded doses of dexamethasone on endogenous myostatin mRNA and protein expression in both the absence and presence of a glucocorticoid receptor antagonist (RU-486). These studies demonstrated that dexamethasone increases both...
myostatin mRNA and protein expression in skeletal muscle cells in vitro by upregulating myostatin gene transcription.

**MATERIALS AND METHODS**

**Cloning of the human myostatin promoter.** A human genomic P1-derived artificial chromosome library in pAd10SacBII (DMPC-HFF#1; Genomic System) was screened using a human myostatin random-primed [32P]cDNA probe. Membranes were prehybridized with human Cot I DNA (GIBCO-BRL) and a hybridization mixture (high phosphate buffer: 0.25 M NaCl, 50 mM Na2HPO4, and 2.5% SDS) for 2 h before the probe was added. The filters were incubated at 65°C overnight, washed at a high stringency (0.1% saline-sodium citrate, 1% SDS, 65°C), and autoradiographed. Selected P1 clones, 2m21 and 170f7, were mapped by restriction endonuclease digestions (BamHI, HindIII, SalI, EcoRI, KpnI, EglI, NorI, XbaI, and PstI). The digested DNA was electrophoresed in 0.8% agarose gel, transferred to Hybond N, and washed at a high stringency (0.1% saline-sodium citrate, 1% SDS, 65°C), and autoradiographed. Selected P1 clones, 2m21 and 170f7, were mapped by restriction endonuclease digestions (BamHI, HindIII, SalI, EcoRI, KpnI, EglI, NorI, XbaI, and PstI).

**Transfection.** Plasmid DNA containing one of the myostatin promoter constructs and a PRL-TK plasmid were cotransfected in MBs and cultured in serum-free medium (DMEM) and then incubated at 37°C for a further 20 h. The medium was then replaced with fresh growth medium containing 10% FCS (5% house serum for MTs). At this stage, the various treatment solutions were added. Cell extracts were assayed 48–96 h after transfection using a Dual-Luciferase detection kit, according to the instructions provided by the manufacturer (Promega). Promoter activity was measured using an analytic luminometer TD-20/20 (Turner designs).

**Isolement of nuclear protein extracts.** Nuclear proteins were extracted from C2C12 MBs and MTs using the procedure described by Thai et al. (43). Briefly, cells were harvested by scraping, washed at 0°C in HBSS (10 mM HEPES, 150 mM NaCl, and 1 mM EDTA), collected by centrifugation, and then resuspended in 5 vol of homogenization buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)] with 6 protease inhibitors at 1 µg/ml. The samples were homogenized with 10 strokes of a Dounce pestle B to >60% lysis by microscopic examination. The homogenate was layered on 1 vol of 45% sucrose in homogenization buffer and then centrifuged at 4°C for 15 min at 5,000 g. The nuclear pellet was resuspended in 1 vol of extraction buffer (homogenization buffer plus 0.4 M KCl, 0.2 mM EDTA, and 25% glycerol), placed on ice for 30 min, and centrifuged for 15 min at 4°C. The supernatant containing nuclear proteins was added to a dialysis buffer (15 mM HEPES, 0.5 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol) and was dialyzed in a microdialysis chamber (Pierce). The protein concentration was determined by the biocinchonic acid assay (Pierce), and aliquots were frozen at −70°C until needed.

**Myocyte enhancer factor 2 DNA probe synthesis.** Four specific oligonucleotides were synthesized with the following sequences: myocyte enhancer factor 2 (MEF2)-2T: 5′-GGCGACTAAATAATTTAATAC; MEF2-2B: 5′-GGTGTATTATAATTTATTTAGT; MEF2-1T: 5′-GTGACTTTAAATATTATTG; and MEF2-1B: 5′-GGCTCATATCAAATTTATTTTTAAG. Double-stranded DNA for the MEF2-1A and -2A elements was synthesized by mixing equal molar amounts of the corresponding oligonucleotide pairs (for MEF2-1A: MEF2-1T and -1B and for MEF2-2A: MEF2-2T and -2B) and incubating at 60°C to allow for annealing and finally cooling to 25°C. The resulting double-stranded oligonucleotides had 5′-overhanging ends on both sides, facilitating radiolabeling by a fill-in reaction using the Klenow fragment of DNA polymerase and [α-32P]dCTP. After labeling, the DNA was purified using Sephadex G-25 spin columns (Clontech).

**Gel electrophoretic mobility shift assay.** Protein-DNA binding reactions were performed for 30 min at room temperature by combining 2–8 µg nuclear protein extract with 40,000 counts/min (~1 ng) of DNA probe plus 2 µg poly(dI-dC) (Sigma) in 16 µl of electrophoretic mobility shift assay (EMSA) buffer (15 mM HEPES, pH 7.9, 5 mM MgCl2, 40 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 5% glycerol). For competitive binding experiments, 10- or 50-fold molar excess of two nonlabeled DNA elements were included in the above solution, specifically, F15/R13 (GGATGAAAAACCCACAATTGT), a double-stranded, annealed oligomer containing no sequence homology with MEF2, and F10/R21 (GCTAGCTATGAAACTAAAAAAG), an oligonucleotide containing 7/9 bp homology with the known core MEF2 element (CTA-AATAA). For supershift experiments, 1–2 µl of an antibody specific to the MEF2D transcription factor (Transduction Laboratories) was added to the competitor binding reaction, and the mixture was incubated for an additional 10 min at 4°C.
4°C. Finally, 4 μl of EMSA buffer with 20% glycerol and 0.005% bromphenol blue were added to the reaction mix. A 5% polyacrylamide gel (30:1 bis) containing 5% glycerol in 0.5× TBE (Tris-base 90 mM, boric acid 90 mM, EDTA 2 mM) was prepared, set, and prerun (200 volts at 4°C) for 1 h before the were samples loaded. After 3–4 h of separation at 250 volts, the gel was removed, dried, and exposed to X-ray film for autoradiography.

Analysis of endogenous myostatin mRNA by RT-PCR. Total RNA was extracted from the cultured muscle cells using the TRIzol reagent following the manufacturer’s suggested procedure (GIBCO-BRL). Each sample was DNase treated, and cDNA was synthesized by reverse transcription of 2 μg of total RNA employing an oligo(dT) primer (GIBCO-BRL). Multiplex PCR amplification was carried out on a 2-μl aliquot of the resultant cDNA using the following two primer pairs: rat myostatin (RMst forward: ATG AGG ACA GTG AGA GAG AGG; reverse: GCA CAA GAT GAG TAT GCG G) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (RGap forward: 33/38, GAG ACT GCC ACC CAG AAG ACT; reverse: CAT GCC AGT GAG CTT CCC GGT; see Ref. 15). Thermocycling conditions were denaturation at 94°C for 2 min, amplification (94°C/30 s, 58°C/30 s, 72°C/50 s) for 35 cycles, and a final elongation at 72°C for 10 min. Amplification products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, photographed, scanned, and analyzed using the densitometric software package QuantiScan version 1.5.

Western blot analysis. Protein was extracted from both MB and MT using a denaturing reducing lysis buffer containing 1% SDS, Tris-HCl, and a 1:20 dilution of β-mercaptoethanol. Samples were heat denatured (95°C for 5 min) and electrophoretically separated using 12% Tris-glycine polyacrylamide gels (ReadyGel; Bio-Rad, Hercules, CA), and the proteins were visualized using Coomassie brilliant blue staining. The electrophoretically separated samples were then transferred to a nitrocellulose membrane (Hyperbond-ECL; Amersham) and immunodetected using the previously described procedure (16). The antibody employed was the previously described myostatin polyclonal antibody B (16). An anti-rabbit-IgG secondary antibody linked to horseradish peroxidase (HRP) was used. Blots were developed with an enhanced chemiluminescent substrate for HRP and exposed to film (ECL Hyperfilm; Amersham).

**RESULTS**

Cloning and characterization of the human myostatin promoter. We screened a human genomic P1-derived artificial chromosome library with a full-length myostatin cDNA probe and identified two clones (2m21 and 17017, ~25 and 40 kb in size, respectively) that contained the myostatin promoter. Restriction digestion and Southern blot analysis of the clones identified two fragments of ~5 and 9 kb in size. The 9-kb fragment only hybridized with a probe consisting of the 3’-end of myostatin cDNA. Conversely, the 5-kb fragment only hybridized with a probe comprising the 5’-end of myostatin cDNA, suggesting that it contained the myostatin promoter. Consequently, the 5-kb fragment was subcloned and sequenced. Sequence analysis revealed that this fragment contained not only the 5’-end of myostatin cDNA but also a 3.3-kb 5’-upstream region of the human myostatin gene. Further sequence analysis identified three TATA boxes, a partial CCAAT box, and five octameric sequences homologous to the consensus binding sites of POU homeodomain proteins (Table 1 and Fig. 1). Twelve E boxes corresponding to myogenic differentiation factor 1 (MyoD) binding sites, two regions homologous to MEF2 binding sites, a putative peroxisome proliferator-activated receptor-γ (PPARγ) binding site, and a region homologous to the consensus sequence of the nuclear transcription factor POU proteins

**Table 1. Distribution of selected transcriptional response elements on the human myostatin promoter**

<table>
<thead>
<tr>
<th>Identified Elements</th>
<th>Position(s) from Transcription Initiation Site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATA box</td>
<td>−18/−24, −41/−48, −398/−404</td>
<td>42</td>
</tr>
<tr>
<td>CCAAT box</td>
<td>−86/−92</td>
<td>42</td>
</tr>
<tr>
<td>POU proteins</td>
<td>−660/−667, −712/−719, −1399/−1406, −1883/−1890, −2949/−2956</td>
<td>38</td>
</tr>
<tr>
<td>MYF2-2</td>
<td>−456/−465</td>
<td>7, 34</td>
</tr>
<tr>
<td>MYF2-1</td>
<td>−1335/−1344</td>
<td>3, 12, 25</td>
</tr>
<tr>
<td>PPARγ</td>
<td>−966/−976</td>
<td>44</td>
</tr>
<tr>
<td>NF-κB</td>
<td>−2982/−2997</td>
<td>19</td>
</tr>
<tr>
<td>ARE</td>
<td>−1083/−1097</td>
<td>36</td>
</tr>
<tr>
<td>GRE</td>
<td>−277/287, −807/−621</td>
<td>41</td>
</tr>
<tr>
<td>palGRE</td>
<td>−1083/−1097</td>
<td>41</td>
</tr>
<tr>
<td>tatGRE</td>
<td>−207/−221, −1362/−1372, −2137/−2150</td>
<td>29</td>
</tr>
<tr>
<td>CRE</td>
<td>−195/−202, −2329/−2336</td>
<td>21</td>
</tr>
<tr>
<td>TRE</td>
<td>−130/−143, −966/−976, −3236/−3251</td>
<td>39</td>
</tr>
<tr>
<td>ER6</td>
<td>−1251/−1263, −2465/−2481, −3265/−3277</td>
<td>39</td>
</tr>
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</table>

MEF, monocyte enhancer factor; PPAR-γ, peroxisome proliferator-activated receptor-γ; NF-κB, nuclear factor-κB; ARE, androgen response element; GRE, glucocorticoid response element; palGRE, palindromic GRE; tatGRE, tyrosine aminotransferase GRE; CRE, cAMP response element; TRE, thyroid hormone response element.

**Statistical analysis.** At least three repetitions of each experiment were performed. In each experiment, every treatment was performed in quadruplicate. Values are means ± SE. The statistical significance was determined by one-way ANOVA. All pairwise multiple comparison procedures (Fisher’s least significant difference method) in experiments are shown in Figs. 2 and 5. The statistical significance was determined by one-way ANOVA. All pairwise multiple comparison procedures (Dunnett’s method) in experiments are shown in Fig. 4. All statistical tests were performed using the Jandel SigmaStat statistical software package. P < 0.05 was taken as the level of statistical significance.
factor (NF-κB binding site were also found (Table 1 and Fig. 1).

The proposed promoter sequence contained a number of sites that corresponded to the consensus sequences of various hormone-binding sites, in particular, an androgen response element (ARE), five sequences corresponding to three different glucocorticoid response elements [GRE, palindromic GRE (pal-GRE)], and tyrosine aminotransferase GRE (tatGRE), and three thyroid hormone response elements (TRE; Table 1 and Fig. 1). Also identified were three ER6 sequences that are known to have similar function to TRE (39) and two regions homologous to a cAMP response element (Table 1 and Fig. 1).

Mapping myostatin promoter transcriptional activity. The eight myostatin promoter deletion constructs that we tested displayed differing levels of transcriptional activity in transfected C2C12 (Fig. 2) and L6 cells (data not shown), both at the MB and MT stages. Interestingly, the transcriptional activities of all the constructs, except for pMK6, were approximately twofold higher in MT than in MB (Fig. 2).

In both C2C12 and L6 muscle cell lines, deletions of the 5′-upstream region of the promoter from −3322 to −1187 resulted in a marked increase of transcriptional activity. Of the eight constructs, pMK8 (1187 bp), had the highest transcriptional activity, more than three times that of the longest construct pMK1 (3322 bp). These findings suggest the presence of possible transcriptional inhibitory elements between −3322 and −2062 and −1447 and −1187. Deletions of sequences proximal to −1187 caused loss of transcriptional activity, suggesting the presence of transcriptional enhancers, especially between −1187 and −529. Similarly, as the promoter was deleted even further, to a length of 327 bp (pMK6), there was a decrease of approximately threefold in transcriptional activity, suggesting that the region between −327 and −529 contains a transcriptional enhancer (Fig. 2).

Characterization of MEF2 sites. To test whether the two MEF2 elements identified on the myostatin promoter sequence could bind MEF2 nuclear proteins, we performed gel shift experiments using synthetic DNA probes for MEF20-1A and -2A and nuclear extracts from C2C12 MB and MT. High-molecular-weight protein-DNA complexes were formed with both MEF2 DNA sequences, but MEF2-2A bound much more strongly than MEF2-1A to proteins in both MB and MT. We observed approximately sevenfold more prominent protein-DNA complexes being formed with

Fig. 1. Sequencing analysis of the myostatin promoter. The transcriptional starting site was located 115 bp upstream from start codon ATG. The promoter consists of three TATA boxes (boxed) located at positions −18/−24, −41/−48, and −398/−404. Sequences homologous to CCAAT box, POU protein octamers, glucocorticoid response elements (GRE), palindromic GRE (palGRE), tyrosine aminotransferase GRE (tatGRE), androgen response element (ARE), cAMP response element (CRE), E box, thyroid hormone response element (TRE), myocyte enhancer factor 2 (MEF2), peroxisome proliferator-activated receptor-γ (PPARγ), and nuclear factor (NF-κB) elements are underlined.
MEF2-2A in MT than in MB extracts (Fig. 3A). The specificity of protein-DNA binding was demonstrated by complete competition of complex formation by a 50-fold excess of the MEF2-2A sequence and partial competition by MEF2-1A (Fig. 3B). Partial competition was also observed with the F10/R21 oligonucleotide pair, which fortuitously has a small MEF2-like DNA sequence. No competition was observed with F15/R13 DNA. Furthermore, the antibody specific for MEF2D was able to supershift up to 50% of the protein-DNA

Fig. 3. Autoradiograph of the electrophoretic mobility shift assay (EMSA) of MEF2 DNA elements with nuclear proteins from C2C12 cells. A: DNA probes for MEF2-1A or MEF2-2A elements were incubated with C2C12 cell nuclear extracts from myoblasts (MB) and myotubes (MT) in varying amounts (μg) or with no protein (0). The resulting protein-DNA complexes (arrow at top) were separated from any unbound DNA probe. B: DNA competition and antibody supershift EMSA. Double-stranded competitor DNA fragments were added in 10- or 50-fold molar excess to reactions containing 5 μg of MT nuclear protein. Binding with the DNA probe for MEF2-1A is shown on bottom, and binding with the DNA probe for MEF2-2A is shown in top. Supershift experiments are shown in the four lanes marked with asterisks. Reactions with 1 or 2 μl of antibody (Ab) MEF2D or nonspecific Ab IgG are indicated. Control (0) is the reaction with no antibody. Supershifted complexes of Ab + MEF2D are shown.
complexes (Fig. 3B), indicating that these complexes contain the MEF2D transcription factor. The non-shifted complexes may contain other MEF2 factors (e.g., MEF2B). The binding of MT MEF2 transcription factors specifically with the MEF2-2A element in the myostatin promoter is consistent with its postulated function in activation of this muscle-specific enhancer, since deletion of this promoter region causes a significant loss of the promoter activity.

Glucocorticoid agonist dexamethasone increases transcriptional activity of myostatin promoter in vitro. The presence of sequences homologous to the consensus sequence of the GRE suggested that glucocorticoids might regulate myostatin transcription. To test this hypothesis, we transfected the myostatin promoter construct pMK1 into both C2C12 and L6 cells and measured the changes of its transcriptional activity after 96 h. Dexamethasone dose dependently increased the luciferase reporter activity in C2C12 and L6 muscle cell lines (Fig. 4). A concentration of 1 nM dexamethasone had minimal effect on transcriptional activity, whereas maximal induction was observed at 100 nM dexamethasone (Fig. 4, A and B). Increasing dexamethasone concentration, >100 nM, did not further increase myostatin transcriptional activity (data not shown).

To determine whether the effects of dexamethasone on myostatin gene transcription were mediated through a glucocorticoid receptor-mediated pathway, we incubated C2C12 muscle cells with 100 nM dexamethasone (Fig. 4, A and B). Increasing dexamethasone concentration, >100 nM, did not further increase myostatin transcriptional activity (data not shown).

Because multiple GREs (41) were found distributed through the whole myostatin promoter, we tested which of these response elements was functioning by measuring the transcriptional activity of various myostatin promoter deletion constructs that had been transfected into L6 cells and incubated with 100 nM dexamethasone (Fig. 5). Myostatin transcriptional activity was significantly increased in all constructs except pMK2. Interestingly, we observed a significant increase in myostatin transcriptional activity by dexamethasone, even in the shortest construct [pMK6 (327 bp)]. This suggests that the regulatory elements necessary for mediating the response to dexamethasone are present in the proximal 327 bp of the promoter region. Although responsiveness to dexamethasone was retained by the pMK6 construct containing only 327 bp of the 5'-upstream regulatory region, the over-
all transcriptional activity of this construct was significantly lower than that of other constructs containing longer segments of the promoter, indicating that elements proximal to this region might be necessary for the optimal transcriptional response to dexamethasone. Construct pMK8 had the highest increase in transcriptional activity (~3-fold that of the control) after dexamethasone treatment, whereas construct pMK2 appeared to have very little influence (Fig. 5).

**Dexamethasone increases endogenous myostatin mRNA and protein expression.** To determine whether dexamethasone affects the expression of endogenous myostatin mRNA and protein, we incubated C2C12 cells with dexamethasone (100 nM), dexamethasone (100 nM)-RU-486 (1,000 nM), and RU-486 (1,000 nM) alone for 4 days. Northern blot did not detect the presence of myostatin RNA in any of the cell cultures (data not shown). However, RT-PCR analysis indicated that incubation with dexamethasone increased myostatin mRNA expression approximately two- to three-fold over the basal level (Fig. 6). This effect was nullified in cells that were incubated with dexamethasone and RU-486 (Fig. 6). Addition of dexamethasone also resulted in a significant increase in the 30-kDa myostatin immunoreactive protein, as determined by Western blot analysis (Fig. 6). As with mRNA, the effect of dexamethasone on myostatin immunoreactive protein was antagonized by concurrent incubation with RU-486. Incubation with RU-486 alone had no significant effect on myostatin mRNA or protein expression.

**DISCUSSION**

We characterized the 3.3-kb 5’-flanking regulatory region of the human myostatin gene and found it to contain the consensus DNA sequences of a number of transcription factor response elements. Among those identified were GRE, ARE, MEF2, PPARγ, and NF-κB, suggesting that the corresponding proteins may influence the expression of myostatin during the regulation of muscle growth.

Of particular interest was the identification of multiple E boxes. These elements, which are present in the control regions of many skeletal muscle structural genes (4, 9, 27, 47), have been shown to be activated by certain myogenic factors, especially MyoD (6, 24). MyoD, a member of the basic helix-loop-helix (bHLH) superfamily of proteins (33, 34), is required to generate the MB population and thus is instrumental in specifying the skeletal muscle lineage (37). The presence of E boxes in the upstream region of our promoter suggests that myostatin expression may result as a response to MyoD expression.

Our studies showed that the elements necessary for both the basal transcription of myostatin, and its response to glucocorticoids, are present within the proximal 327 bp of the promoter region. Further information on the roles of the different regions of the promoter was provided by the deletion construct experiments that indicated that the promoter contained two regions that were capable of inhibiting transcription (between −3322 and −2062, and −1447 and −1187) and an area with probable transcriptional enhancers (between −1187 and −529). The removal of an MEF2 site (between −529 and −327 bp) caused a threefold decrease in transcriptional activity, suggesting that the region acts as a strong transcriptional enhancer.

MEF2 is a four-member gene family, MEF2A through MEF2D (25), that encodes nuclear proteins belonging to the MADS (MCM1, agamous, deficiens, serum) superfamily of DNA-binding proteins (48). In the adult mouse, MEF2C transcripts are restricted to skeletal muscle, brain, and spleen (28, 29), whereas MEF2A, MEF2B, and MEF2D are expressed ubiquitously (48). The MEF2 enhancer has been found in the regulatory regions of many muscle structure genes (26, 35). Because the MEF2 proteins are involved in complex heterotypic protein-protein interactions with members of the myogenic bHLH family (32), it has
been suggested that they might play a role in stabilizing myogenin expression during mammalian myogenesis (13). Recently, it was found that MEF2 and the myogenic bHLH proteins act within a regulatory network that establishes the differentiated phenotype of skeletal muscle (14, 23, 34). Indeed, expression of MEF2 genes marks the development of early myogenic lineages during embryogenesis (10, 14), whereas the trans-activation function of the MEF2 proteins is required for muscle-specific gene expression and differentiation during mammalian myogenesis (1). The presence of MEF2 binding sites on the myostatin promoter and its strong enhancing effect shown on the transcriptional activity suggest that MEF2 is influential in myostatin expression during various stages of skeletal muscle growth.

We demonstrated that myostatin expression could be regulated in two in vitro skeletal muscle models and that the transcriptional activity of the promoter was lower in MB cells than in MT. Furthermore, endogenous myostatin mRNA could only be detected in MT, suggesting that myostatin expression in cultured MB is very low. A possible explanation for this observation may be found in the results of the gel shift assay, which showed that a much higher density band of protein-DNA complexes was generated with MT nuclear extract than in MB. This suggests that there is a higher concentration of MEF2 or related proteins in MT, which in turn may lead to a greater enhancement of the myostatin promoter activity and consequently a higher expression of myostatin.

Glucocorticoids, widely used for the treatment of a number of inflammatory disorders, are well known to induce muscle atrophy in humans and experimental animals. Previous in vitro and in vivo studies have suggested that the effects of glucocorticoids on muscle may be mediated through the inhibition of protein synthesis and an increase in protein degradation. However, the precise molecular mechanisms by which glucocorticoids induce the catabolic effects on muscle remain unknown.

Our data constitute the first demonstration that a glucocorticoid, dexamethasone, can upregulate both myostatin mRNA and protein concentrations in skeletal muscle cells in vitro. The results suggest that the dexamethasone-induced increase in myostatin mRNA expression may be due, at least in part, to the induction of myostatin gene transcription. Furthermore, the effect of dexamethasone was antagonized by the glucocorticoid receptor antagonist RU-486, and the addition of the mineralocorticoid agonist fludrocortisone failed to influence myostatin transcription or mRNA and protein expression. It can therefore be surmised that dexamethasone acts upon myostatin via an as yet unspecified glucocorticoid receptor-mediated mechanism. Also, we do not know whether dexamethasone has additional posttranscriptional or posttranslational effects on myostatin.

The shortest of our deletion constructs, containing only 327 bp of the proximal region, was found to be sufficient for mediating the myostatin transcriptional response to dexamethasone. It is possible that two GREs, tatGRE at −221 and a partial GRE at −287, might mediate these responses to dexamethasone. However, the magnitude of the transcriptional response to glucocorticoid administration was significantly greater in promoter constructs containing the region between −2062 and −1187. This region contains numerous GREs. Taken together, these data suggest that the coordinated action of multiple GREs might be needed for the optimal response to dexamethasone to be attained.

Our studies demonstrate that glucocorticoids may induce muscle atrophy through the upregulation of myostatin gene expression. We have previously reported that recombinant myostatin inhibits muscle cell replication and protein synthesis in vitro. Therefore, it is possible that an increase in myostatin expression resulting from glucocorticoid administration may cause a decrease in protein synthesis that in turn might lead to a loss of muscle mass. This hypothesis needs to be tested in vivo.

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REFERENCES


